

# Carbohydrate as covalent crosslink in human inter- $\alpha$ -trypsin inhibitor: a novel plasma protein structure

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The primary structure of inter- $\alpha$ -trypsin inhibitor is partially elucidated, but controversy about the construction of the polypeptide backbone still exists. We present evidence suggesting that inter- $\alpha$ -trypsin inhibitor represents a novel plasma protein structure with two separate polypeptide chains covalently crosslinked only by carbohydrate (chondroitin sulphate)

Inter- $\alpha$ -trypsin inhibitor; Carbohydrate, Chondroitin sulfate; Crosslink; Primary structure; Polypeptide chain

## 1. INTRODUCTION

Inter- $\alpha$ -trypsin inhibitor (ITI) is a serine protease inhibitor present in human serum and plasma (0.4–0.5 g/l) [1]. The primary structure of ITI has recently been reviewed [2] as a single polypeptide chain of  $M_r$  180000 with the inhibitory activity located near the N-terminal. The N-terminal amino acid sequence of ITI is identical to the sequence found in inhibitory active ITI metabolites normally present in plasma (ITI-derivatives) and urine (UTI) [3]. UTI and ITI derivatives are assumed to be produced in vivo by limited proteolysis of ITI in the N-terminal region of the polypeptide chain [3]. A protease responsible for these cleavages has not been identified, and high concentrations of proteases are generally needed for in vitro fragmentation of ITI [4].

Covalently bound glycosaminoglycan (chondroitin sulphate) has been identified in purified UTI [5]. Digestion of UTI ( $M_r$  44000) with chon-

droitinase (EC 4.2.2.4) removes the glycosaminoglycan without proteolytic modification, giving rise to UTI c ( $M_r$  26000) [6].

The hypothesis that ITI is comprised of a single polypeptide chain has been challenged [7] by the detection of mRNA from baboon liver, with translation products corresponding to two immunochemically distinct polypeptide chains both related to ITI. cDNA clones from human liver libraries with nucleic acid sequences coding for well-known amino acid sequences of ITI has recently been isolated [8–10]. One of these clones contains the complete sequence of UTI [9] terminated by a stop codon. These results indicate that human ITI is not a single chain structure but composed of at least two polypeptide chains, one of which is identical to the polypeptide chain of UTI.

It is generally accepted, however, that ITI migrates as an undissociable molecule in SDS-PAGE even after treatment with reducing agents [2]. Thus, if more than one polypeptide chain is present in ITI, a covalent link different from a disulphide bridge must exist between the chains.

In this paper we present evidence suggesting that

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inter- $\alpha$ -trypsin inhibitor represents a novel plasma protein structure with two polypeptide chains covalently crosslinked only by carbohydrate (chondroitin sulphate).

## 2. MATERIALS AND METHODS

### 2.1. Reagents and chemicals

#### 2.1.1. Enzymes

Ovine testicular hyaluronidase (EC 3.2.1.35, Calbiochem no.38594, spec. act. 6775 N.F. units/mg), bovine testicular hyaluronidase (LEO Denmark no.001412, spec. act. >100 N.F. units/mg), hyaluronidase from *Streptomyces hyalurolyticus* (Sigma no.1136) and chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4, Sigma no.2905, spec. act. 1.5 units/mg).

#### 2.1.2. Purified proteins

Human ITI (2 mg/ml) was purified from fresh normal EDTA plasma by polyethyleneglycol precipitation, anion-exchange chromatography and gel filtration (unpublished). Separation of ITI fragments generated by hyaluronidase digestion was achieved by gel filtration on a TSK G3000SW column at a flow rate of 0.4 ml/min in 0.1 M  $\text{CH}_3\text{COONH}_4$ , pH 7.0. Human serum albumin from Kabi Vitrum, Stockholm, Sweden. Rabbit anti-human ITI from Dakopatts, Denmark (code A 301). Molecular mass standards were rabbit muscle myosin (200 kDa),  $\beta$ -galactosidase (116 kDa), rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa).

#### 2.1.3. Protease inhibitors

Benzamidine hydrochloride (Sigma no.B-6506), *p*-chloromercuribenzoic acid (Sigma no.C-4378), phenylmethylsulfonyl fluoride (Sigma no.P-7626), 6-aminohexanoic acid (Merck Art.800145), EDTA (Merck Art 8418).

### 2.2. Enzymatic digestions

Enzymatic digestions were performed at 37°C with 5 vols normal human serum or purified ITI mixed with 3 vols reaction buffer and 2 vols enzyme preparation. Aliquots were withdrawn after 10, 20, 40, 80 and 160 min and frozen immediately until analysed. Reaction buffer contained 20 mM EDTA, 0.25 M NaCl, 0.1 M  $\text{CH}_3\text{COONa}$ , pH 4.8, in hyaluronidase digestions, and 20 mM EDTA, 0.25 M NaCl, 0.25 M Tris, 0.18 M  $\text{CH}_3\text{COONa}$ , 0.05% human albumin, pH 8.0, in chondroitinase digestions.

Enzyme preparations were preincubated 30 min, 25°C in 0.1 M  $\text{CH}_3\text{COONa}$ , 0.25 M NaCl, pH 4.8, containing the protease inhibitors 25 mM benzamidine hydrochloride, 20 mM phenylmethylsulfonyl fluoride, 2.5 mM *p*-chloromercuribenzoic acid and 50 mM 6-aminohexanoic acid. Enzyme concentrations during preincubation were 50 N.F. units/ml for ovine testicular hyaluronidase, 25 N.F. units/ml for bovine testicular hyaluronidase, 31 N.F. units/ml for hyaluronidase from *Streptomyces hyalurolyticus* and 3.3 U/ml for chondroitinase ABC.

### 2.3. Immunoelectrophoresis and SDS-PAGE

Crossed immunoelectrophoresis according to Høiby and Axelsen [11] and crossed-line immunoelectrophoresis according to Krøll [12] were performed in Tris/barbital buffer, pH 8.6, against rabbit anti-human ITI, 1.4  $\mu\text{l}/\text{cm}^2$ .

### 2.4. Solubilization of immunoprecipitates

Immunoprecipitates were isolated from unstained crossed immunoelectrophoresis. Precipitates were cut out separately, eluted 180 min at 37°C in a buffer containing 3% SDS and 40 mM dithioerythritol. The eluates were concentrated in Amicon B 15 concentration cells before application in SDS-PAGE.

### 2.5. Amino terminal sequence determination

Automatic sequencing was performed on a protein sequencer (Applied Biosystems, model 477 A) using the program 'NORMAL-1' supplied with the instrument. The released phenylthiohydantoin amino acid derivatives were identified and quantified by on-line HPLC analysis (Applied Biosystems, model 120 A) on a reversed-phase column (C 18).

## 3. RESULTS AND DISCUSSION

### 3.1. Enzymatic digestion of ITI in serum

The immunoprecipitation pattern of ITI in normal human serum is shown in fig.1a. The main precipitate represents intact ITI, and the minor with a higher electrophoretic mobility represents ITI derivatives [14]. This precipitation pattern remained unchanged when serum was analysed after incubation for 160 min at the reaction conditions applied in the enzymatic digestions but without added enzyme.

Normal human serum digested by hyaluronidase from ovine testes (molar ratio: enzyme/ITI ~ 1:50) reveals the ITI degradation pattern shown in fig.1. The precipitate of intact ITI gradually decreases with the concomitant appearance of two new precipitates: one of lower and one of higher electrophoretic mobility. Digestion of intact ITI is completed in 160 min (fig.1f), with two immunochemically non-identical components as end products. This precipitation pattern does not change with further digestion.

In addition to the digestion presented in fig.1, other endoglycosidases with various glycosaminoglycan specificities were examined for their ability to cleave ITI. Hyaluronidase from bovine testes, acting on both hyaluronic acid and chondroitin sulphate [15,16], and chondroitinase ABC, selective for chondroitin sulphate [17], both cleaved ITI with the same result as shown in fig.1. In the

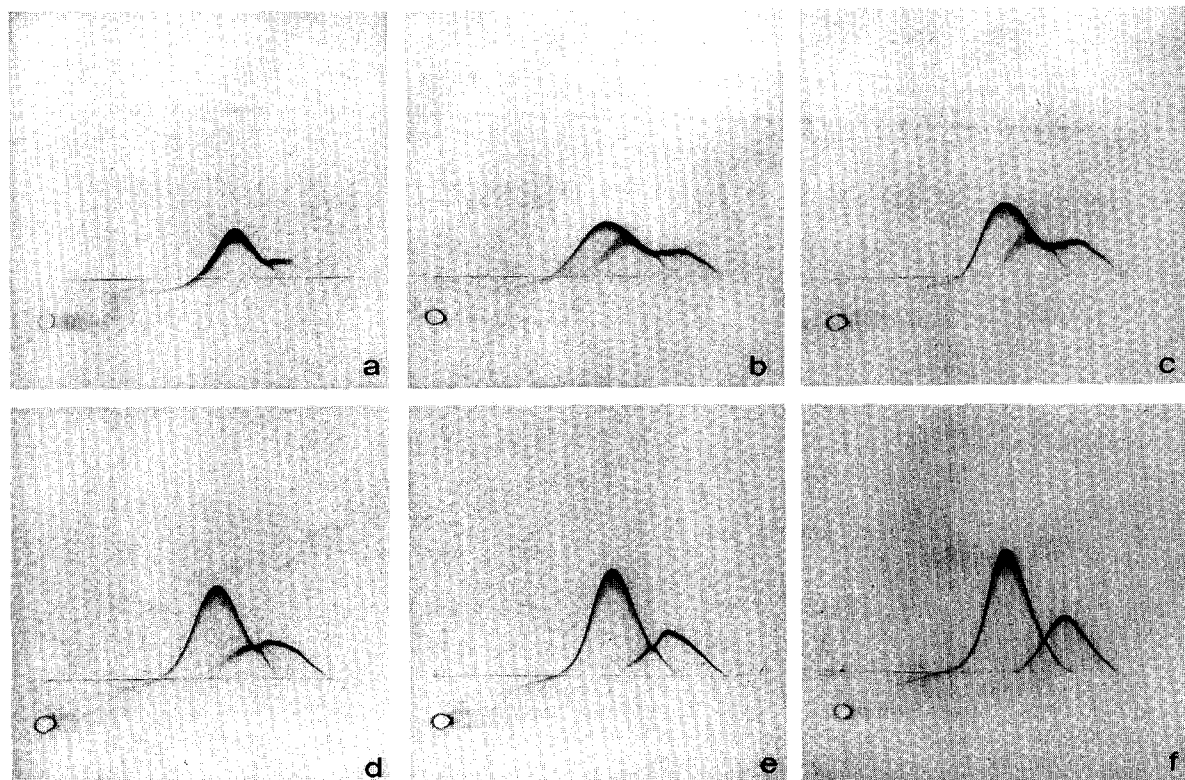


Fig.1. Serum digested by hyaluronidase, analysed in crossed immunoelectrophoresis against anti-human ITI. Samples (15  $\mu$ l): (a) normal human serum diluted 1:2; (b-f) serum digested by hyaluronidase for 10 min (b), 20 min (c), 40 min (d), 80 min (e) and 160 min (f).

presence of chondroitin sulphate ABC (5 mg/ml) these cleavages were inhibited. In contrast to the above mentioned enzymes hyaluronidase from *Streptomyces hyalurolyticus*, selective for hyaluronic acid [18] did not cleave ITI. According to the selectivity of the applied enzymes these results suggest the existence of a chondroitin sulphate crosslinkage between two polypeptide chains in ITI.

### 3.2. Enzymatic digestion of purified ITI

The existence of two polypeptide chains in ITI is further confirmed by digestion of purified ITI with ovine testicular hyaluronidase (fig.2, lanes 3-8). Purified reduced ITI appears in SDS-PAGE as a single band of  $M_r > 200000$  (lanes 3 and 4). As the digestion proceeds, ITI gradually decreases (lanes 5-8) with the concomitant appearance of two new components of  $M_r$  160000 (heavy chain) and  $M_r$  26000 (light chain). A faint transient band of low

mobility (lanes 5-8) is possibly due to partially digested chondroitin sulphate.

An identical pattern was observed when the serum samples from the digestion shown in fig.1 were subjected to SDS-PAGE and subsequent immunoblotting using a polyclonal anti-human ITI as primary antibody (not shown).

The relation between the heavy/light chain in SDS-PAGE and the slow/fast migrating components in immunoelectrophoresis is verified in fig.2 (lanes 10-12). The applied samples are solubilized immunoprecipitates representing intact ITI (lane 10), slow migrating component (lane 11) and fast migrating component (lane 12). Intact ITI appears to have the same molecular mass as purified undigested ITI. The slow migrating component and the heavy chain generated by enzymatic digestion are apparently identical according to SDS-PAGE. The supposed relationship between the fast migrating component and the

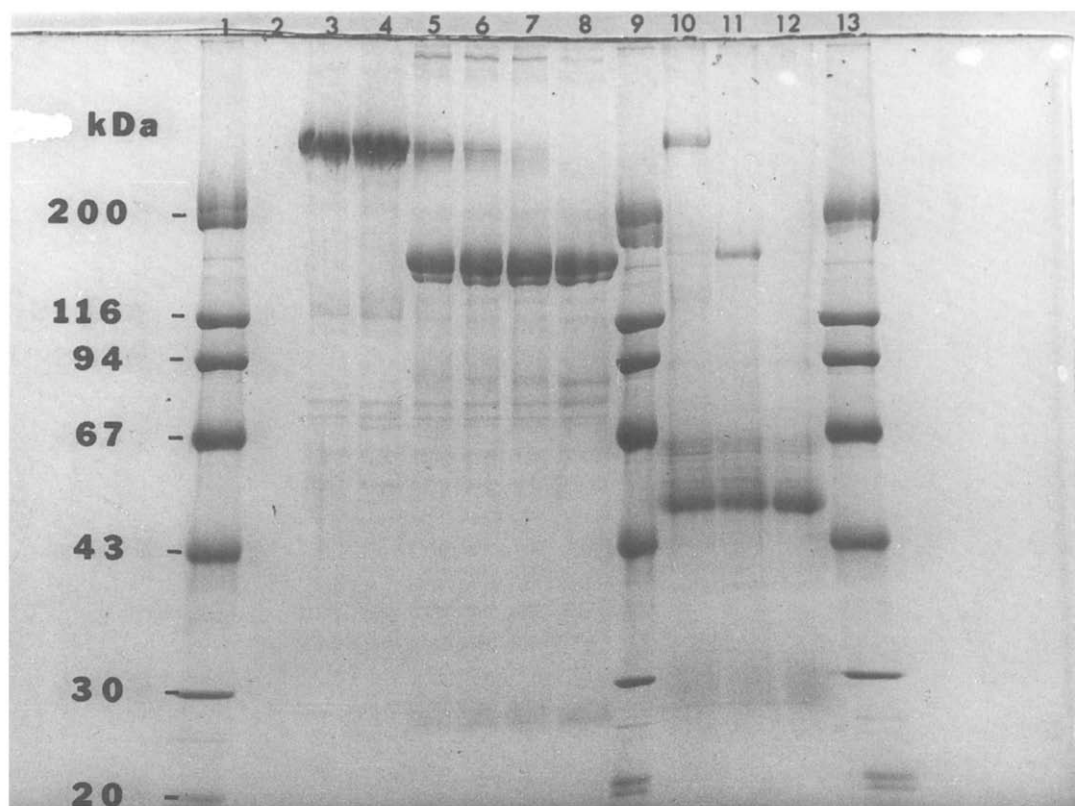


Fig.2. SDS-PAGE of purified ITI and solubilized immunoprecipitates before and after digestion with testicular hyaluronidase. Lanes: 1, molecular mass standards; 2, testicular hyaluronidase (same amount as in lanes 5–8); 3, purified ITI; 4, purified ITI incubated without added enzyme for 160 min at conditions used in the digestion; 5–8, ITI digested by testicular hyaluronidase for 10 min (5), 20 min (6), 40 min (7) and 160 min (8); 9, molecular mass standards; 10, solubilized immunoprecipitate of intact ITI; 11, solubilized immunoprecipitate of slow migrating component; 12, solubilized immunoprecipitate of fast migrating component; 13, molecular mass standards. The amount of ITI antigen in lanes 3–8 is 8  $\mu$ g.

light chain cannot be established by this technique, due to the weak intensity of the light chain, which is further masked by a diffuse zone ( $M_r$  25000–30000) originating from the rabbit antibodies (light chains).

### 3.3. Isolation of heavy and light chain

Separation of the heavy and light chain was performed by gel filtration of purified ITI digested by hyaluronidase. Two components of  $M_r$  160000 and 26000 were isolated and their immunochemical relationship with the two components generated in serum are confirmed by crossed-line immunoelectrophoresis (fig.3). Apparently the isolated heavy chain is related to the slow migrating component, and the light chain to the fast migrating component.

If normal human urine is applied to the intermediate gel, a precipitation pattern identical to fig.3b is obtained.

### 3.4. N-terminal sequence determination

In order to finally establish the connection between intact ITI and hyaluronidase-generated heavy chain and light chain, these components were subjected to automatic Edman degradation. The amino acid sequences obtained are shown in table 1.

It is evident that intact human ITI possess two N-terminal sequences present in equimolar amounts. The N-terminal amino acid sequence of the purified heavy chain and light chain completely account for the entire double sequence obtained for ITI. The N-terminus of the light chain is iden-

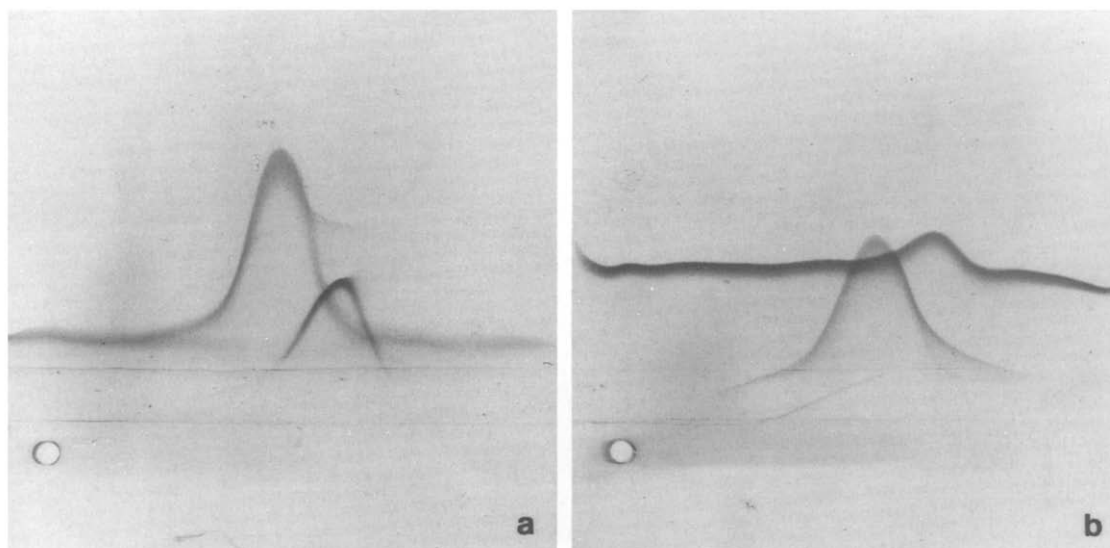


Fig.3. Crossed-line immunoelectrophoresis illustrating immunochemical relationship between the isolated heavy/light chain of ITI, and the fast/slow migrating component. Samples applied in the wells are serum digested by hyaluronidase (as in fig.1f). Intermediate gels contained 3  $\mu$ g purified heavy chain (a) or 4  $\mu$ g purified light chain (b).

tical to that of UTI [3] as far as residue number 20, where sequencing was terminated.

Based upon the selectivity of the applied enzymes, and on our analysis of the generated reaction products, we propose that human ITI is composed of two distinct polypeptide chains, one

light chain and one heavy chain. The two chains are covalently crosslinked by chondroitin sulphate, and the light chain seems to be identical to the polypeptide chain of UTI. We know of no other plasma protein where two polypeptide chains are covalently connected exclusively by carbohydrate. The physiological significance of this structural feature is unknown.

Table 1

N-terminal amino acid sequences obtained from intact ITI and purified ITI-fragments generated by hyaluronidase digestion

Residue number	1	2	3	4	5	6	7	8	9	10	11	12
Intact ITI	A	V	L	P	Q	E	E	E	G	—	G	G
	S	L	P	G	E	K	—	Q	A	V	D	T
									M	M	E	—
Light chain	A	V	L	P	Q	E	E	E	G	—	G	G
Heavy chain	S	L	P	G	E	K	E	Q	A	V	D	T
									M	M	E	—

Roughly equimolar amounts of PTH derivatives were recovered at step 1, 2 and 5 in the continuous double sequence obtained from intact ITI. Amino acid sequence analysis of isolated heavy chain as well as heavy chain forming part of ITI, unambiguously identified two residues in step 9–11, whereas the preceding steps stated a single residue only. Repetitive yields of amino acid PTH derivatives were 92% (light chain) and 96% (heavy chain). (—) Indicates that no obvious PTH amino acid, other than those stated, were recovered in the steps concerned

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